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PHYTOCHEMICAL PROFILING AND ANTIOXIDANT ASSESSMENT OF CASSIA AURICULATA LEAVES VIA GC-MS

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ABSTRACT
Background: This research aimed to investigate the phytochemical composition, quantify key bioactive compounds such as phenolics and flavonoids, analyze the secondary metabolite profile using GC-MS on methanolic leaf extracts, and evaluate the antioxidant capacity via the DPPH assay. **Methodology:** The Soxhlet extraction method is employed to obtain crude extracts of *Cassia auriculata* using solvents including ethanol, methanol, chloroform, and water. These extracts undertook qualitative analysis to detect various bioactive phytochemicals. The total phenolic and flavonoid concentrations were quantified. The methanolic leaf extract underwent phytochemical analysis using a gas chromatography-mass spectrometry (GC-MS) device, following established procedures. The antioxidant capacity of the methanolic leaf extracts was assessed by determining their ability to scavenge free radicals of 2,2-diphenyl-1-picrylhydrazyl. **Results and Discussion:** Initial phytochemical screening revealed the presence of various secondary metabolite groups. Out of all the solvent extracts assessed, the methanolic extract displayed the highest concentrations of phenolic and flavonoid compounds, measuring 9.48 ± 0.06 mg of Gallic acid equivalents per gram of extract and 6.56 ± 0.03 mg of Quercetin equivalents per gram of extract, respectively. GC-MS analysis of the methanolic extract identified 28 bioactive compounds with known pharmacological significance. **Conclusion:** The antioxidant activity, evaluated using the DPPH radical scavenging assay, demonstrated that the methanolic extract had the most potent radical scavenging effect among the extracts tested (IC50-48.96 µg/ml). These findings suggest that *Cassia auriculata* leaves extract is a promising source of natural antioxidants and bioactive compounds, supporting its traditional use in herbal medicine.

INTRODUCTION

India's rich heritage of conventional medicine streams, including Ayurveda, Siddha, and Unani, has employed the therapeutic potential of numerous medicinal plants. The non-toxic nature and affordability of these plant-based remedies have fueled ongoing interest in exploring and scientifically validating their bioactive compounds [1–4]. Focusing on medicinal plants, *Cassia auriculata*, a member of the Fabaceae family and commonly referred to as "Tarwad" in Maharashtra,

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is a shrub that is extensively utilized in various traditional medicinal practices. Its flowers are customarily used to treat skin conditions and control body odor. Moreover, multiple parts of this plant are used to address a range of health issues, including conjunctivitis, diabetes, and rheumatism, underscoring its broad therapeutic potential. The bark is valued for its astringent properties, while the leaves and fruits are used for their anthelmintic activity. The seeds have been utilized in managing eye disorders, and the roots are applied in the treatment of skin diseases [5,6]. Previous studies have documented the medicinal properties of Cassia auriculata, including antidiabetic, hypolipidemic, Anticancer, Antioxidant, Antipyretic, and hepatoprotective effects [7–9]. *Cassia auriculata* leaves and flowers have demonstrated antidiabetic and antilipemic properties, suggesting potential benefits in managing diabetes and coronary heart disease [10]. Moreover, phytochemicals extracted from *Cassia auriculata* leaves have shown significant in vitro cytotoxicity against HCT 15 colon cancer cell lines [11].

A separate study focusing on extracts of flowers revealed that extracts of *Cassia auriculata* flowers, prepared using ethanol and methanol, exhibit notable antioxidant properties. Flavonoids are possibly responsible for this impact, and tannins, which are proficient at neutralizing ABTS radical cations and DPPH radicals in vitro [12]. Natural antioxidants derived from plants have shown notable capacity to inhibit free radicals, which can otherwise cause cellular damage and contribute to the onset of Chronic diseases such as diabetes, cancer, arthritis, and cardiovascular disorders. Phenolic and flavonoid compounds, in particular, have been identified as crucial agents in the neutralization of free radicals [13,14]. The methanolic extract of *Cassia auriculata* leaves, as determined through Phytochemical screening, has been verified to contain alkaloids, flavonoids, triterpenoids, and glycosides [15].

Comparatively, given the diverse therapeutic potential and the presence of valuable bioactive compounds, this research aims to conduct a qualitative examination of the phytochemical components, characterize the volatile secondary metabolites using GC-MS, quantify the concentrations of phenolics and flavonoids, and assess the antioxidant potential of Cassia auriculata leaf extract. Furthermore, this study will lay the groundwork for identifying pharmacologically active secondary metabolites and their promising therapeutic benefits.

MATERIAL AND METHODS

Collection of Plant and Preparation of Leaf Extract

The *Cassia auriculata* Fresh leaves were primarily collected in November from Phaltan Tehsil, India (17°59'00.3"N 74°21'13.9"E). The Authorized person from the SBR college Botany Department identified and confirmed the plant. After meticulously washing the collected leaves with fresh water to remove any dirt or debris, they were allowed to air-dry in the shade for about a week. Using a mortar and pestle, the leaves were ground into a fine powder when they had completely dried.

Approximately 50 grams of the dried leaves powder were subjected to extraction with ethanol, methanol, chloroform, and double-distilled water using a Soxhlet apparatus. For each solvent, 50 grams of powder is used. Extraction was performed at temperatures ranging from 55°C to 80°C, ensuring continuous hot percolation over 24 hours [16,17]. The extract obtained was filtered and concentrated at 40°C under reduced pressure using a rotary evaporator, resulting in a thick, semi-solid, dark brown residue. This residue was then stored in airtight containers and kept in a deep freezer. This extract will be utilized for qualitative analysis of plant metabolites, quantitative analysis of total phenols and total flavonoids, GC-MS profiling, and evaluation of antioxidant capacity through the DPPH method [18].

Phytochemical Screening (Qualitative Analysis)

An initial qualitative assessment was carried out on the leaf extracts of *Cassia auriculata* to identify the presence of critical bioactive constituents. These extracts, obtained using methanol, ethanol, chloroform, and water, were subjected to standard phytochemical analysis techniques [19]. The qualitative screening was designed to detect the presence of various phytochemicals, including secondary metabolites [19,20]. Thereby providing essential insights into the potential medicinal properties of *Cassia auriculata* leaf extracts.

QUANTITATIVE ESTIMATION OF TOTAL PHENOLIC AND TOTAL FLAVONOIDS CONTENT

Estimation of total phenolic content

The Folin-Ciocalteu method was employed to determine the total phenolic content [16,21] with some adjustments. An ethanolic extract solution at a concentration of 1 mg/ml was prepared for the analysis. A 1 mL sample of either the extract or the gallic acid standard solution was placed into a 25 mL volumetric flask containing 9 mL of distilled water. A reagent

blank was prepared using distilled water. Then, 1 ml of Folin-Ciocalteu phenol reagent was added to the mixture and stirred. After 5 minutes, 10 ml of 7% Na_2CO_3 solution was introduced, and the volume was adjusted to the mark. The mixture was incubated for 90 minutes at room temperature, and the absorbance was measured at 550 nm against a reagent blank using a UV-visible spectrophotometer. The total phenolic content was expressed in terms of mg gallic acid equivalents (GAE). The absorbance of the test samples was measured and recorded in triplicate.

Estimation of Total Flavonoid Content

The total flavonoid content was assessed using the aluminum chloride colorimetric method [16]. A 1 mL sample of either the extract or quercetin standard solution was added to a 10 mL volumetric flask containing 4 mL of distilled water. Next, 0.30 ml of 5% NaNO2 was added, followed by 0.3 ml of 10% AlCl3 after five minutes. Another 5 minutes later, 2 mL of 1 M NaOH was added, and the solution was topped up to 10 mL with distilled water. The mixture was then stirred, and the absorbance was recorded at 510 nm against a blank. The total flavonoid content was quantified as milligram Quercetin Equivalents (QE). The absorbance of the test sample was measured and recorded in triplicate to reduce error, ensure accuracy, and reproducibility.

GC-MS Analysis

Methanol solvent is well known for its ability to effectively extract a wide range of phenolic and flavonoid compounds from leaf extracts. The chemical composition and metabolite profiling of *Cassia auriculata* methanolic leaf extract were characterized using Gas Chromatography-Mass Spectrometry (GC-MS) performed on a Shimadzu QP 2010 system (Tokyo, Japan), equipped with an RTX-1 fused silica capillary column and an AOC-20i auto-sampler. A 3.0 µl aliquot of the sample was injected in split mode using a 10 µl syringe, with the injector temperature maintained at 250°C. Ultrapure helium gas was used as the carrier gas at a constant flow rate of 2.0 mL/min through the GC column. The oven's temperature was set to start at 50°C, held for 2 minutes, followed by a ramp at 10°C per minute up to 300°C, with a final hold of 8 minutes. The transfer line temperature to the mass selective detector (Thermal Aux 2) was set at 250°C. Mass spectra were recorded over a mass-to-charge (m/z) range of 35 to 800. Compound identification was performed by comparing the obtained spectra against the National Institute of Standards and Technology 14 mass spectral database [22].

Evaluation of In Vitro Antioxidant Activity

In vitro DPPH Free Radical Scavenging Assay in 96-well plates is employed for extracts of *Cassia auriculata*. The Antioxidant capabilities of *Cassia auriculata* leaf extracts in methanol, ethanol, and water were assessed for their ability to neutralize DPPH (1,1-Diphenyl-2-picryl-hydrazyl) free radicals, employing the method described [23]. Stock solutions of both the crude extracts and the reference antioxidant, ascorbic acid, were formulated in methanol at a concentration of 1 mg/ml (calculated on a dry weight basis). These stock solutions were then further diluted with methanol to create five distinct concentrations: 20, 40, 60, 80, and 100 µg/ml. Aliquots of 100 µl of each test concentration were added to wells of a microtiter plate, followed by the addition of 100 µl of 0.1% methanolic DPPH solution. The mixtures were then incubated in the dark at room temperature for 30 minutes. A change in colour from purple to yellow indicated effective free radical scavenging, while a pale pink colour suggested weaker activity. All assays were performed in triplicate. Methanol served as the blank control. Absorbance measurements were taken at 517 nm using an ELISA plate reader. The DPPH radical scavenging activity or % RSA was determined using the formula [23]:

$$\textit{DPPH radical scavenging activity}\ (\%) = \frac{\textit{Optical density of control} - \textit{Optical density of sample}}{\textit{Optical density of control}} \times 100$$

Statistical analyses

Statistical analyses of data were performed using Freeware PAST version 4.1 [24] and Microsoft Excel. The IC_{50} values were calculated from the dose-response curves, and the results are presented as the mean ± standard deviation (SD). Microsoft Excel was used for data organization, preliminary calculations, and graphical representation.

RESULTS AND DISCUSSION

The qualitative analysis revealed that methanolic and ethanolic extracts of *Cassia auriculata* leaves contained a wide range of phytoconstituents, including alkaloids, flavonoids, phenolics, tannins, saponins, and glycosides (Table 1). Methanolic extracts, in particular, showed a notably higher presence (++ marking) of flavonoids and phenolics, which are widely recognized for their antioxidative and therapeutic roles [25-29]. These findings are consistent with those of a study reported by

Monisha et al. (2017) [15], who reported a similar broad-spectrum phytochemical profile in *Cassia auriculata* leaves, affirming the species' richness in bioactive compounds.

Quantitative estimation of Total Phenolics and Flavonoids contents of *Cassia auriculata* Leaves extracts

As summarized in Table 2, methanol proved to be the most effective solvent, extracting the highest concentration of total phenolics (9.48 ± 0.06 mg GAE/g) and flavonoids (6.56 ± 0.03 mg QE/g). Ethanol and water yielded moderate and lower concentrations, respectively. These outcomes corroborate the findings of Patil et al. (2023)[16], who emphasized the superior polarity and extraction efficiency of methanol for phenolics and flavonoids from plant matrices. Statistical analyses validated the significance of solvent impact, with a one-way ANOVA confirming variation in extract yields ($p = 0.0455$). Post hoc analysis revealed a significant difference between methanol and aqueous extracts ($p = 0.0325$), highlighting the solvent's crucial role in maximizing recovery of bioactive metabolites. Similar trends have been observed in other medicinal plants, such as Psidium guajava (Patil et al., 2023) [16] and Hypochaeris radicata (Senguttuvan et al., 2014) [17], suggesting that methanol remains the solvent of choice for maximizing the yield of antioxidant phytoconstituents.

Table 1: Phytochemical constituents of various extracts of Cassia auriculata leaves

Phytochemicals	Extracts			
	Methanolic	Ethanolic	Chloroform	Aqueous
Alkaloids	+	+	+	+
Glycosides	+	+	+	+
Flavonoids	++	++	-	+
Phenolics	++	++	-	+
Tannins	+	+	-	+
Saponins	+	+	+	+
Carbohydrates	+	+	+	+
Reducing Sugar	+	+	+	+
Proteins	-	-	-	-

Key: + = present, ++ = high amount present, - = not present

Table 2: Total phenolics and flavonoids content of various extracts of Cassia auriculata leaves

Extraction solvent	Total phenolics contents (mg GAE/g ±SD)	Total Flavonoids contents (mg QE/g ±SD)
Methanol	9.48 ± 0.06	6.56±0.03
Ethanol	5.48 ± 0.08	3.64±0.04
Aqueous	1.78 ± 0.07	1.44±0.05

The values are expressed as Mean ± SD of triplicates

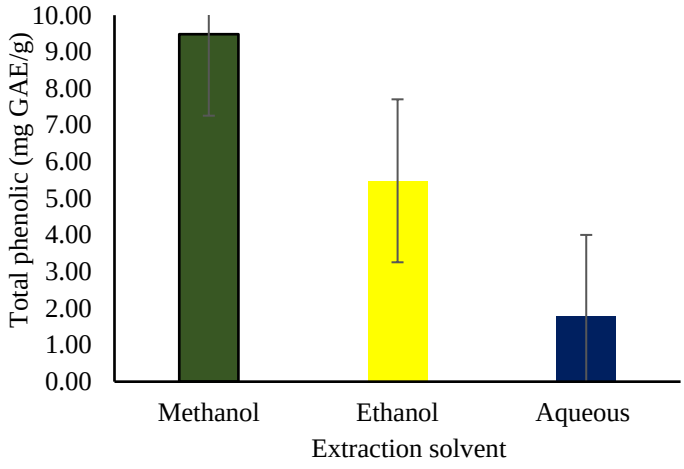

Figure 1: Total phenolic content of different extracts of *Cassia auriculata* leaves

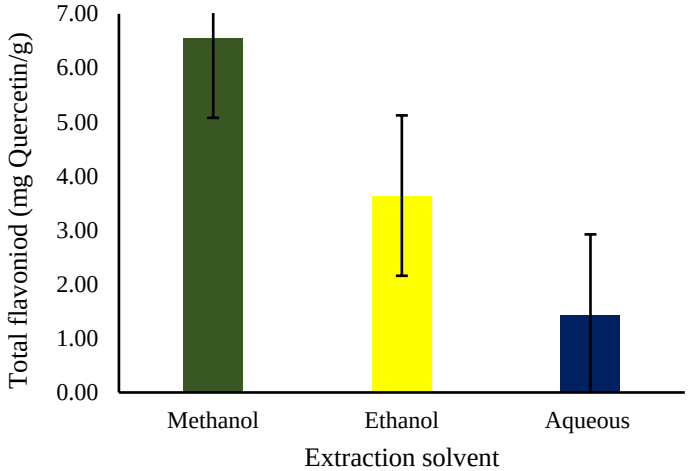

Figure 2: Total flavonoids content of different extracts of *Cassia auriculata* leaves

Table 3: Phytoconstituents of Cassia auriculata methanolic leaves extract identified and quantified by GC-MS

Peak	RT	RI	Peak Area %	Height %	Compound name	Molecular Formula	Suggests Potential Pharmocological Activity
1	15.21	1285	0.25	0.41	Tetradecane	C14H30	Antioxidant,Antimicrobial [33]
2	15.99	1518	0.52	0.58	Quinoline, 1,2-dihydro-2,2,4 trimethyl-	C12H15N	Antioxidant [34]
3	16.78	1555	0.76	1.06	2,4-Di-tert-butylphenol	C14H22O	Anti-inflammatory,Antimicrobial and Antioxidant [35]
4	18.84	2109	0.54	0.97	Heneicosane	C21H44	Antiasthmatics, Urine acidifiers and Antimicrobial [35]
5	19.31	1746	0.29	0.40	Heptadecane, 8-methyl-	C17H36	Antimicrobial,Antioxidant [36]
6	19.58	1769	3.93	3.54	Tetradecanoic acid,methyl ester	C13H26O2	Antioxidant,Antibacterial [33]
7	20.34	0	0.29	0.52	Neophytadiene	C20H38	Anti-inflammatory, Antimicrobial, Antioxidant,[37]
8	20.94	2009	0.35	0.46	Eicosane	C20H41	Antibacterial, Antifungal Cytotoxic [35]
9	21.31	2081	0.65	0.67	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-dien	C17H24O3	No activity reported
10	21.62	4765	20.05	18.01	l-(+)-Ascorbic acid 2,6-dihexadecanoate	C38H68O8	Antioxidant, Cardioprotective [38]
11	22.86	0	0.31	0.45	Dotriacontane, 1-iodo-	C32H66	No activity reported
12	23.05	2045	0.34	0.42	Phytol	C20H40O	Antimicrobial, Anti-inflammatory Anticancer, Diuretic [39]
13 13	23.32	2101	1.94	1.73	9,12,15-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	Anti-inflammatory, AntiCancer, Antioxidant[40]
14	23.48	2167	7.93	8.58	Octadecanoic acid	C17H35COOH	Antibacterial,Antifungal, Antitumoral [35]
15	23.86	2574	0.90	1.51	1-Docosanol, acetate	C24H48O2	Antiviral-9 [41]
16	26.42	2498	22.8	22.58	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl ester)	C19H38O4	Antimicrobial [42] Antioxidant,nematicidal [43]
17	26.99	0	0.26	0.46	Triacontane, 1-iodo-	C30H61I	Antibacterial activity[37]
18	27.81	2923	4.12	5.85	4,4'-((p-Phenylene)diisopropylidene)diphenol	C24H26O2	Antioxidant [44]
19	27.97	2681	11.75	10.94	Octadecanoic acid, 2,3-dihydroxypropylester	C21H42O	Anticancer,Antibacterial [45]
20	18.73	1714	10.52	3.39	4-O-Methylmannose	C7H14O6	Antiinflamatory [46]
21	16.78	1555	0.46	0.82	Phenol, 2,5-bis(1,1-dimethylethyl)	C14H22O	Antioxidant, Antimicrobial [33]
22	21.62	1968	13.31	15.33	n-Hexadecanoic acid	C16H32O2	Antibacterial, Antifungal [33]

RT= Retention Time, RI = Retention Index

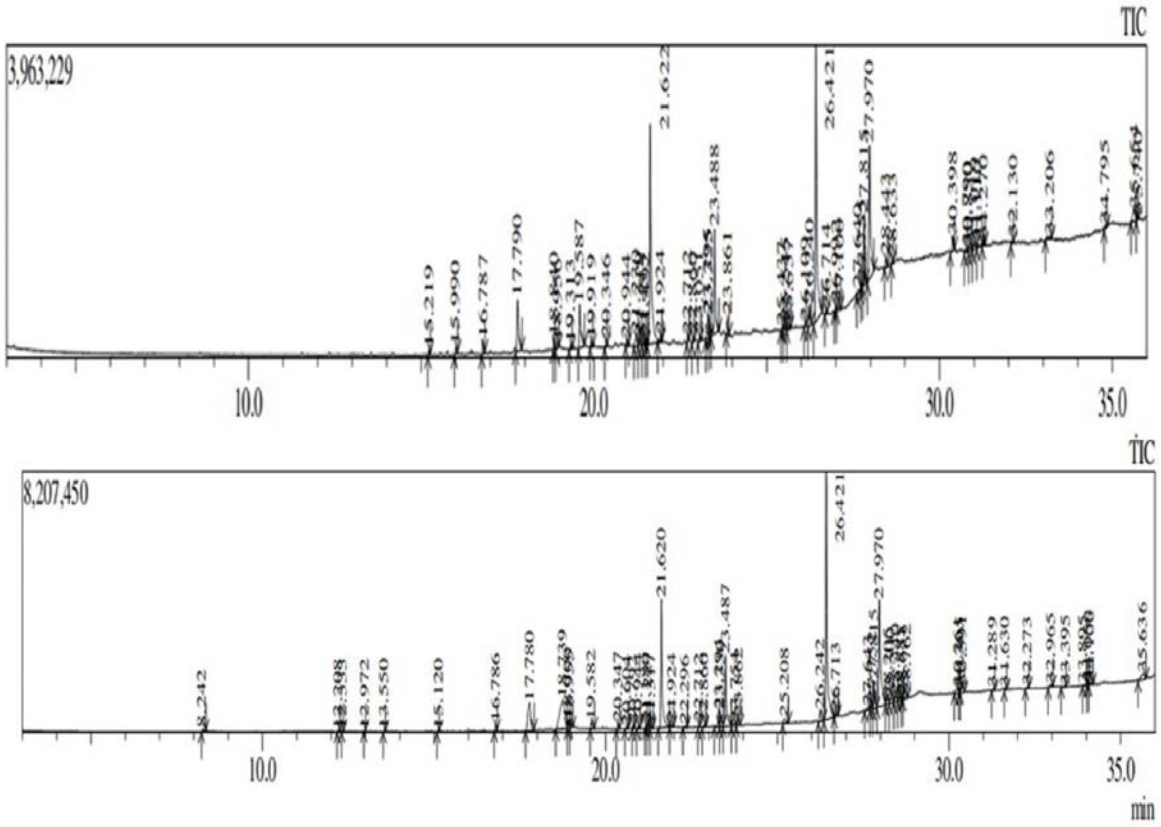

Figure 3: GC-MS Chromatogram of Cassia auriculata methanolic leaves extract

Table 4: DPPH free radical scavenging activity of different extracts of Cassia auriculata leaves extract

Extraction Solvent	DPPH Scavenging % (% Radical Scavenging Activity)					IC50
Methanol	38 (±1)	46.33 (±2.52)	55.33 (±0.58)	68.00 (±4)	71.00(±1)	48.96 µg/ml
Ethanol	25 (±2)	32.33 (±2.52)	42.33 (±1.53)	48.33 (±2.52)	55.67 (±2.52)	93.15 µg/ml
Aqueous	18 (±3)	25.00 (±2)	31.67 (±0.58)	36.00 (±2)	41.33 (±1.53)	193.94 µg/ml
Ascorbic Acid Standard	55 (±1)	68.67 (±3.06)	83.00 (±2)	90.00(±2)	97.67(±0.58)	32.02 µg/ml

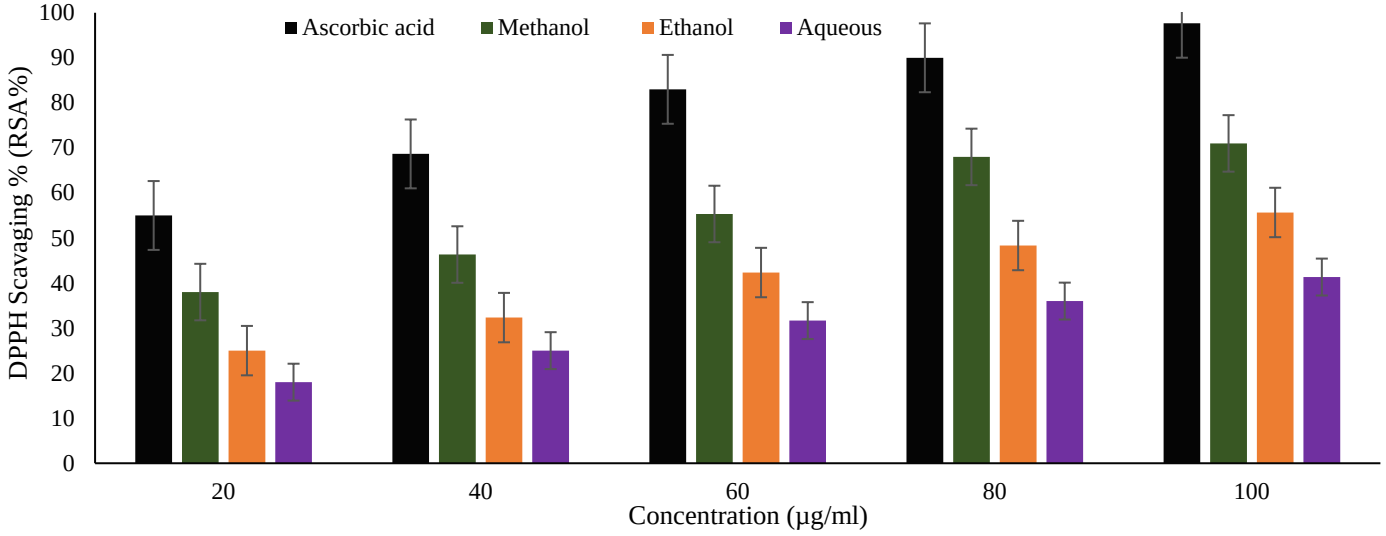

Figure 4: Antioxidant Assay by DPPH scavenging activity of Cassia auriculata leaves extract, Ascorbic acid as standard

GC-MS analysis led to the identification of 28 compounds (Table 3, Fig. 3) encompassing various phytochemical classes with known pharmacological relevance. Major constituents included Hexadecanoic acid, 2-hydroxy-1-hydroxymethyl ester (22.8%), l-(+)-Ascorbic acid 2,6-dihexadecanoate(20.05 %), n-Hexadecanoic acid (13.31%), and Octadecanoic acid, 2,3-dihydroxypropyl ester (11.75%). These molecules have been previously documented for their antioxidant, antimicrobial, anti-inflammatory, and cardioprotective properties (Mazumder et al., 2020; Shah et al., 2023) [30,31], Kumar et al., 2021 [32].

The detection of l-(+)-Ascorbic acid ester, a potent antioxidant, strengthens the evidence of the extract's free radical scavenging potential. Moreover, the presence of neophytadiene, phytol, and 2,4-di-tert-butylphenol aligns with reports by Akter et al. (2024) [37] and Cupido et al. (2022) [35], who highlighted similar compounds in *Lagerstroemia thorelli* and *Opuntia megarrhiza*, respectively, as contributors to significant biological activities.

The antioxidant activity, as assessed by the DPPH assay, confirmed the superior radical scavenging ability of the methanolic extract with an IC_{50} of 48.96 µg/ml, outperforming both ethanol (93.15 µg/ml) and aqueous (193.94 µg/ml) extracts. Although the standard antioxidant, ascorbic acid, showed a lower IC_{50} (32.02 µg/ml), the methanolic extract's performance still reflects a strong antioxidant capacity (Table 4, Figure 4). A positive correlation was observed between the antioxidant activity and the concentration of total phenolics and flavonoids, consistent with previous studies, such as those by Ismail et al. (2017) [13] and Sharma et al. (2022) [47], Mahato et al. (2017) [48], which have documented that phenolic-rich plant extracts exhibit robust DPPH scavenging effects. Comparative analysis with similar investigations further validates these findings. For instance, *Dryopteris cochleata* leaves (Kathirvel & Sujatha, 2016) [18] and *Gomphrena globosa* flowers (Esmat et al., 2020) [20] both demonstrated notable antioxidant efficacy via the DPPH assay, with GC-MS analysis confirming the presence of bioactive phenolics and flavonoids, paralleling the phytochemical and antioxidant synergy observed in this study.

The alignment of the identified phytoconstituents with bioactivities such as antioxidant, anticancer, anti-inflammatory, and antimicrobial effects provides a pharmacological rationale for the traditional use of *Cassia auriculata* in herbal medicine. The high antioxidant potential of the methanolic extract, supported by GC-MS data and phytochemical quantification, positions this plant as a promising candidate for nutraceutical and therapeutic applications.

CONCLUSION

In conclusion, the methanolic leaves extract of *Cassia auriculata* exhibited the maximum levels of Total phenolics and flavonoid compounds amongst the solvents tested. DPPH Antioxidant Assay verified that the Antioxidant activity of the methanolic extract significantly surpassed that of the ethanolic and aqueous extracts. A strong connection was observed between the total phenolic and flavonoid content and the antioxidant capacity, underscoring methanol's Potency as a solvent for extracting bioactive phytochemicals. Furthermore, GC-MS profiling reveals the presence of 28 distinct secondary metabolites with recognized or suggested pharmacological potential. These findings support further detailed investigations to isolate these bioactive compounds and evaluate their potential therapeutic applications.

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AUTHOR CONTRIBUTION

All authors made equal contributions to this research. Abhishek Manoj Ranaware was responsible for reviewing the literature, conducting the experiments, interpreting the findings, and drafting the initial manuscript. Savita Pravin Nalawade oversaw the study and offered technical support in completing the manuscript draft. The final version of the manuscript has been read and approved by all authors.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

FINANCIAL ASSISTANCE

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